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Article

An Ecological Network of Polysaccharide Utilization among Human Intestinal Symbionts

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Summary

Background: The human intestine is colonized with trillions of microorganisms important to health and disease. There has been an intensive effort to catalog the species and genetic content of this microbial ecosystem. However, little is known of the ecological interactions between these microbes, a prerequisite to understanding the dynamics and stability of this host-associated microbial community. Here we perform a systematic investigation of public goods-based syntrophic interactions among the abundant human gut bacteria, the Bacteroidales.
Results: We find evidence for a rich interaction network based on the breakdown and use of polysaccharides. Species that utilize a particular polysaccharide (producers) liberate polysaccharide breakdown products (PBPs) that are consumed by other species unable to grow on the polysaccharide alone (recipients). Cross-species gene addition experiments demonstrate that recipients can grow on a polysaccharide if the producer-derived glycoside hydrolase, responsible for PBP generation, is provided. These producer-derived glycoside hydrolases are public goods transported extracellularly in outer membrane vesicles allowing for the creation of PBP and concomitant recipient growth spatially distant from the producer. Recipients can exploit these ecological interactions and conditionally outgrow producers. Finally, we show that these public goods-based interactions occur among Bacteroidales species coresident within a natural human intestinal community.
Conclusions: This study examines public goods-based syntrophic interactions between bacterial members of the human gut microbial ecosystem. This polysaccharide-based network likely represents foundational relationships creating organized ecological units within the intestinal microbiota, knowledge of which can be applied to impact human health.

Introduction

The human intestine is home to a diverse and complex microbial ecosystem composed of trillions of bacteria. This microbial community provides many benefits to the host [1–3] and is also implicated in numerous diseases [1–3]. High-throughput compositional and metagenomic analyses have provided a catalog of the members and genes within the human intestine [4–7]; however, we know little about the relationships among these bacteria. As the intestinal microbiota is critical to human health, it is essential to understand the ecology of this habitat, i.e., the abiotic and biotic determinants that dictate the composition and dynamics of individuals and groups.

The colon harbors the greatest diversity and number of bacteria in the human body, with more than 90% of the members belonging to two phyla: the Bacteroidetes and the Firmicutes [4, 5, 8]. The gut Firmicutes are distantly related to each other and comprise different classes and orders [4, 5, 8]. In contrast, the human gut Bacteroidetes belong to one order of closely related members, the Bacteroidales, with three dominant genera, the *Bacteroides*, the *Parabacteroides*, and the *Prevotella*. Individual strains and species of Bacteroidales are highly abundant and coexist in the human gut at densities of 10^9–10^{10} colony-forming units (cfu)/g feces [4, 9]. In addition, the Bacteroidales are significantly more stable both over the lifetime and across generations of humans [4]. Thus, the question arises as to what allows both the stability of the Bacteroidales and the coexistence of related species.

Microbes perform social behaviors whereby an individual (the actor) performs a function that affects another individual's (the recipient's) fitness, i.e., the ability to reproduce [10]. Cooperative behaviors have a positive effect on the fitness of the recipient and are operational during quorum sensing, biofilm formation, and iron scavenging. Central to these cooperative behaviors is the provision of public goods by the actor such as the secretion of autoinducers, polymers, and siderophores, which are resources available for the benefit of both producing and nonproducing members [10]. Such traits have been shown to be important within clonal populations [10]; however, the impact of these behaviors on closely related microbial species, especially those comprising natural communities [11, 12], is poorly understood. Given the importance of the gut Bacteroidales to human health and the coexistence and stability of numerous closely related species, we sought to determine whether public goods-based interactions exist among these species.

The success of the Bacteroidales in the human gut is in large part due to their ability to utilize diet-derived polysaccharides, many of which arrive to the colon undigested by human enzymes. Bacteroidales members have differing abilities to use these various plant polysaccharides [13–16]. As carbohydrates are critical for survival and thus serve as valuable currency, we investigated whether polysaccharide utilization generates public goods that may impact community stability in an ecosystem subject to variable nutrients—in particular, whether carbohydrate breakdown products are liberated by bacteria able to grow on a particular polysaccharide and whether bacteria unable to live on that polysaccharide can utilize these public goods. We demonstrate a rich and diverse polysaccharide utilization network based on the release and use of public goods. This is the first study to address public goods among the predominant Gram-negative bacteria of the human gut and is one of the first to study these interactions among bacteria from a naturally occurring intestinal community.

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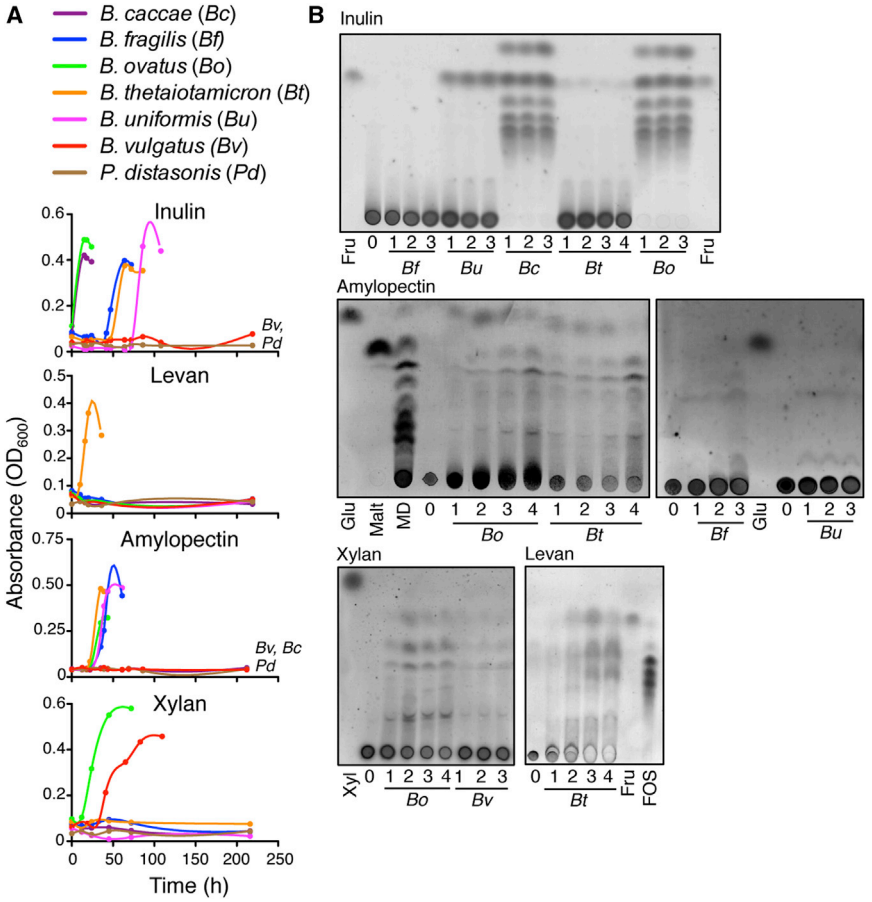

Figure 1. Variation among Bacteroidales Members in Ability to Utilize Polysaccharides and to Publicly Liberate PBPs

(A) Growth curves of bacteria in defined media with indicated polysaccharide as carbohydrate source.

(B) Thin-layer chromatography (TLC) analyses of polysaccharide breakdown products in the CM of primary utilizers through the growth phases (1, early; 2, mid; 3, late log; 4, stationary) in defined media with indicated polysaccharide as carbohydrate source. The intact polysaccharides do not migrate from the bottom of the TLC plate. PBPs are continually generated as they are consumed during bacterial growth due to continued breakdown of polysaccharide. Fru, fructose; FOS, fructose oligosaccharides (derived from inulin); Glu, glucose; Malt, maltose; MD, maltodextrin.

Data are representative of at least three independent experiments; one representative experiment is shown. See also Figure S1.

Results

Polysaccharide Utilization and Liberation of Polysaccharide Breakdown Products by Bacteroidales Members

We screened seven type strains of human gut Bacteroidales (*Bacteroides caccae*, *B. fragilis*, *B. ovatus*, *B. thetaiotaomicron*, *B. uniformis*, *B. vulgatus*, and *Parabacteroides distasonis*) in defined medium with a single polysaccharide as the sole carbohydrate source to identify members that are able (utilizer) or unable (nonutilizer) to grow (Figure 1A). Using four different polysaccharides (the fructose polymers inulin [β1,2] and levan [β2,6], the xylose polymer xylan [β1,4] and the glucose polymer amylopectin), we expanded upon previous studies [13–15] demonstrating that after growth in standard medium, certain Bacteroidales are able to utilize specific polysaccharides for growth, while others cannot (Figure 1A). Five of the strains grew with inulin as the carbon source and two did not (*B. vulgatus* and *P. distasonis*). For amylopectin, there were near equal numbers of utilizers and nonutilizers, whereas xylan is only utilized by *B. ovatus* and *B. vulgatus* and levan only by *B. thetaiotaomicron* [15].

We next investigated whether growth of these polysaccharide-utilizing members creates polysaccharide breakdown products (PBPs) potentially available to members unable to utilize the polysaccharide alone (potential recipients). Therefore, we determined whether PBPs were present in supernatant after growth of a utilizer. This analysis revealed not only that PBPs are liberated extracellularly during growth of Bacteroidales, but also unexpected diversity in PBP liberation by different primary utilizers (Figure 1B). For example, *B. caccae* and *B. ovatus* grown in inulin liberate the monosaccharide fructose and a variety of oligosaccharides, whereas *B. uniformis* liberated only fructose, *B. thetaiotaomicron* only trace amounts of fructose, and *B. fragilis* liberated no detectable PBPs (Figure 1B). Similarly, although all amylopectin utilizers released some form of PBP, growth in amylopectin resulted in qualitative and quantitative differences, with *B. ovatus* and *B. thetaiotaomicron* liberating significant amounts of PBPs and *B. fragilis* and *B. uniformis* very little. Growth of *B. ovatus* and *B. vulgatus* on xylan resulted in liberation of oligosaccharides but not xylose, previously described for *B. ovatus* during growth in xylan [17], whereas *B. thetaiotaomicron* grown in levan liberated both fructose and oligosaccharides (Figure 1B). Therefore, polysaccharide utilization typically results in the production of freely available PBP, but the profiles vary drastically both qualitatively and quantitatively depending on the Bacteroidales utilizer and the polysaccharide.

Utilization of Liberated PBP Public Goods by Non-Polysaccharide-Utilizing Bacteroidales

To identify potential recipients that may utilize producer-derived PBPs, we screened nonutilizing strains for growth when cultured in filter-sterilized conditioned medium (CM) derived from growth of a PBP-producing primary utilizer (for inulin, *B. ovatus*; for levan, *B. thetaiotaomicron*; for amylopectin, *B. ovatus*; and for xylan, *B. ovatus*) (Figure S1 available online). This strategy revealed several nonutilizing bacteria that were able to grow in these CM (Figure 2A). Growth occurred in CM harvested from various phases of growth of the primary utilizer (Figure S2A). Growth of nonutilizers, however, was not universal, and was specific to both polysaccharide and bacteria, as certain nonutilizing species grew readily

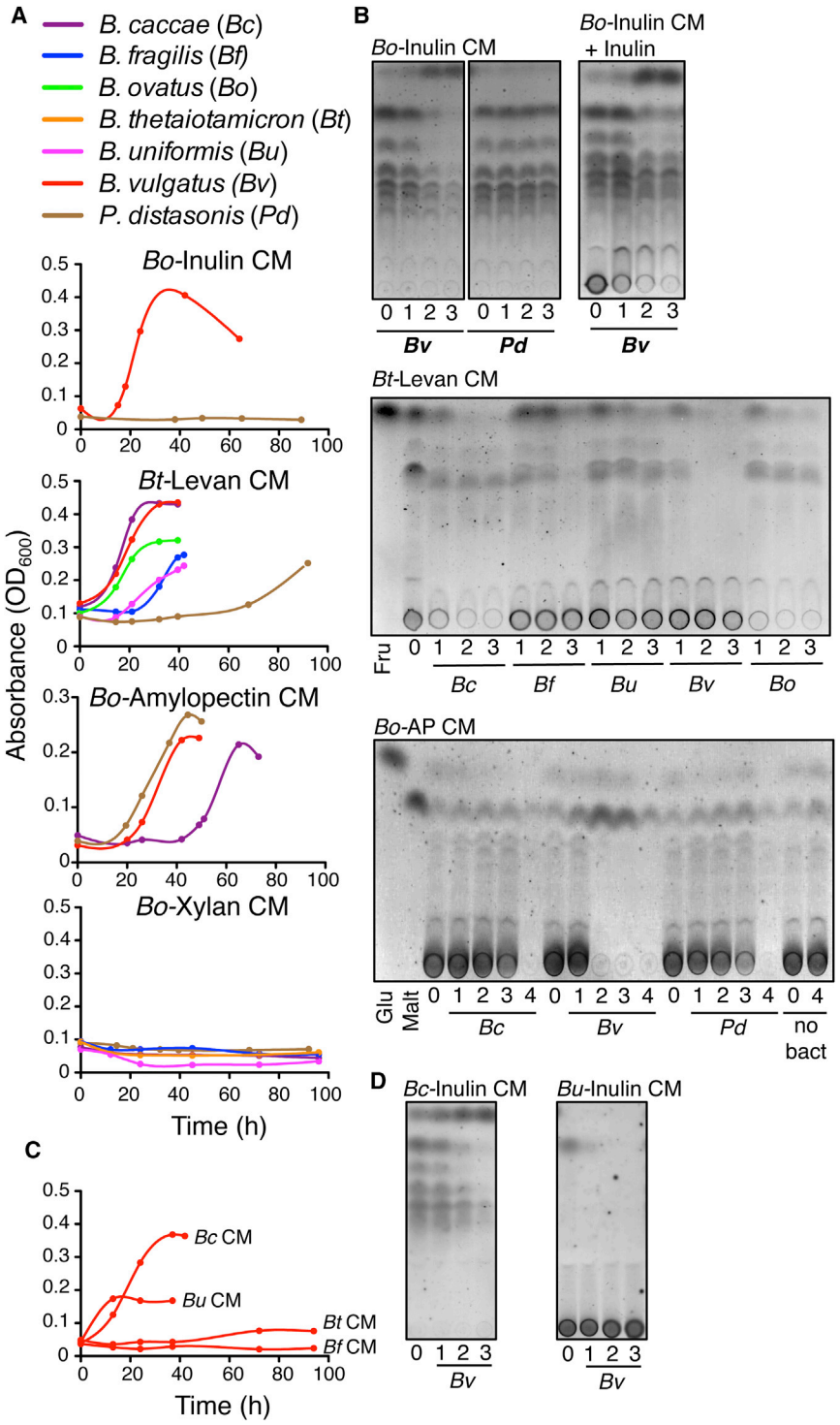

Figure 2. Specific Non-Polysaccharide-Utilizing Bacteroidales Members Can Utilize Liberated PBP Public Goods

(A) Growth curves of non-primary-utilizing bacteria in media conditioned by PBP-liberating utilizers.

(B) TLC analysis of culture supernatants of recipient bacteria grown in the CM of primary utilizers. As *P. distasonis* did not grow in *Bo*-inulin CM, its time points correspond to the growth points of *B. vulgatus* in the same CM through the growth phases (1, mid; 2, late log; 3, stationary).

(C) Growth curves of *B. vulgatus* in inulin media conditioned by different species of inulin-utilizing Bacteroidales.

(D) TLC analysis of culture supernatants of *B. vulgatus* grown in *B. caccae* and *B. uniformis* inulin CM through the growth phases (1, mid; 2, late log; 3, stationary).

Data are representative of at least three independent experiments; one representative experiment is shown. See also Figure S2.

in various CM (termed "recipients"), while others demonstrated no or delayed growth (termed "nonrecipients" and "poor recipients," respectively). Of the inulin nonutilizers, *B. vulgatus* grew in inulin medium conditioned by *B. ovatus*, but *P. distasonis* did not. All species that were unable to grow in levan or amylopectin media grew in levan medium conditioned by *B. thetaiotaomicron* and in amylopectin medium conditioned by *B. ovatus*. In contrast, no xylan nonutilizers were able to grow in xylan medium conditioned by *B. ovatus* (Figures 2A and S2). Furthermore, some recipients grew in the CM early (*B. caccae*, *B. vulgatus*, and *B. ovatus* in *B. thetaiotaomicron* levan CM; *B. vulgatus* and *P. distasonis* in *B. ovatus* amylopectin CM), whereas other recipients grew later (*P. distasonis* in *B. thetaiotaomicron* levan CM; *B. caccae* in *B. ovatus* amylopectin CM) (Figure 2A).

Analysis of PBP profiles during recipient growth revealed that the producer-derived PBPs are depleted from the CM during recipient growth but not when nonrecipients were placed in the CM (Figure 2B), thus demonstrating PBPs as public goods. PBPs are also utilized by producers, as shown by the depletion of PBPs when *B. ovatus* is grown in its own inulin-derived CM and by the depletion of liberated PBPs by *B. ovatus* when inulin is limited (Figures S2B and S2C). The CM of *B. fragilis* grown in inulin, which does not contain detectable PBPs, does not support the growth of recipients (Figure 2C), further demonstrating that PBPs are pubic goods utilized by recipients. Recipient bacteria consumed the different types of PBPs liberated by primary utilizers, and their growth was limited by the quantity of these PBPs (Figures 2C and 2D). Lack of growth by nonrecipients was not due to inhibitory factors or the effects of pH, as all nonrecipients grew when glucose was added to the CM (Figure S2D). Together, these data reveal that the liberation of PBPs is a trait that varies among polysaccharide-utilizing Bacteroidales. In addition, PBPs are not universally used by non-polysaccharide-utilizing members, allowing for the designation of recipients and nonrecipients.

Addition of Producer-Derived Polysaccharide Glycoside Hydrolase/Polysaccharide Lyase Genes to Recipient Bacteria

In Bacteroidales, polysaccharide utilization depends on the presence of clusters of genes termed polysaccharide utilization loci (PULs), each of which encodes products for the utilization of a specific polysaccharide/glycan [16, 18, 19]. PULs typically encode a surface protein that binds the polysaccharide, surface glycoside hydrolases/polysaccharide lyases (GH/PLs) that cleave the large polymer to smaller units, an outer membrane protein that imports these cleaved units to the periplasm, periplasmic glycoside hydrolases that degrade the oligosaccharides to monosaccharides, and regulatory proteins [14, 20]. The ability of recipients to grow on producer-derived PBPs suggested that they contain all the machinery to utilize a particular polysaccharide except for the surface GH/PLs. To explore this possibility, we cloned the genes encoding the *B. ovatus* inulin PLs (BACOVA_04502 and BACOVA_04503), the *B. thetaiotaomicron* levan GH (BT_1760; [15]), and the *B. thetaiotaomicron* amylopectin GH, SusG (BT_3698; [21]) behind a constitutive promoter and placed them in *trans* in recipient strains. Addition of these genes enables recipient bacteria to grow on the primary polysaccharide alone (Figure 3A) and to release PBPs (Figure 3B). Addition of the BT_3698 gene (*susG*) allowed *B. vulgatus* to grow rapidly in amylopectin, but did not support early growth of *B. caccae* (Figure 3A) despite PBP release and availability (Figure 3B), consistent with its delayed growth in amylopectin CM (Figure 2A). Thus, to grow on a particular polysaccharide, recipient bacteria lack only the GH/PLs responsible for initial polysaccharide breakdown and PBP release.

Extracellular Secretion of Producer-Derived GH/PLs

As spatial organization is an important factor in public goods-based interactions between microbes [10], we performed experiments on solid media to characterize dimensional properties of public goods release and utilization. Analysis of the growth of amylopectin utilizers on amylopectin plates, which are opaque due to the optically dense polysaccharide, revealed degradation of polysaccharide in extracellular zones surrounding the bacteria (Figure 4A), indicating that amylopectin is degraded at significant distances from the utilizing bacteria. These zones of degradation would not occur simply by the diffusion of PBPs, but rather require that the GH/PL is secreted from the bacteria. When PBP recipients were plated adjacent to producers, the early growth recipients (*B. vulgatus* on *B. ovatus* amylopectin and inulin plates), but not inefficient/poor recipients (*B. caccae* on *B. ovatus* amylopectin plates), showed growth that was inversely proportional to their distance from the producer (Figure 4A).

These observations raised the intriguing possibility that in addition to PBPs, producer-derived GH/PLs themselves are secreted extracellularly and are public goods liberating PBPs spatially distant from the producer. We first demonstrated that producer-derived CM contains GH/PL activity as revealed by the depletion of polysaccharide and accumulation of PBP over time in cell-free CM (Figure 4B). There was potent inulinase and amylopectinase activity in *B. ovatus*-derived CM, and less xylanase and levanase activity in *B. ovatus* xylan and *B. thetaiotaomicron* levan CM, respectively. *B. fragilis*, which does not liberate PBPs during growth in inulin (Figure 1B), did not yield any detectable inulinase activity in its CM (Figure S3A). To definitively demonstrate that polysaccharide degradation and PBP liberation was due to producer-derived GH/PLs in the CM, we analyzed the CM from recipient bacteria constitutively expressing the GH/PL genes in *trans* grown in defined glucose medium. As these CM are devoid of PBPs at the start of the assay (Figure 4C), the observed PBPs generated upon the addition of

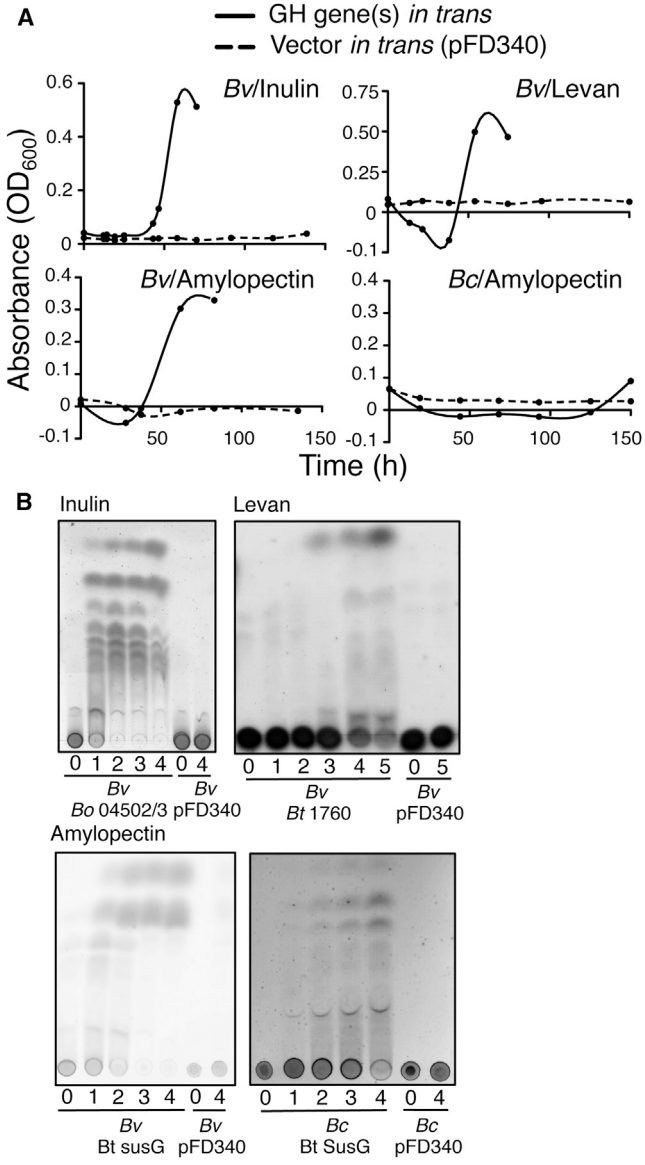

Figure 3. Growth and PBP Liberation by Recipients Containing Producer-Derived GH/PL Genes

(A) Growth curves of recipient bacteria with genes encoding the *B. ovatus* inulin PLs (BACOVA_04502 and BACOVA_04503), the *B. thetaiotaomicron* levan GH (BT_1760), the *B. thetaiotaomicron* amylopectin GH (*susG*, BT_3698), or vector alone (pFD340) in defined polysaccharide media. The initial decrease in OD_{600} in amylopectin and levan media correspond to rapid degradation of these optically dense polysaccharides.

(B) TLC analyses of PBPs released from recipient strains containing GH/PL genes in *trans* or vector alone (pFD340) through the growth phases (Inulin and amylopectin: 1, early; 2, mid; 3, late log; 4, stationary. Levan: 1, lag; 2, early; 3, mid; 4, late log; 5, stationary). As recipient with vector alone did not grow in polysaccharide media, its time points correspond to the growth points of recipient strains containing GH/PL genes in *trans*.

Data are representative of at least independent experiments; one representative experiment is shown.

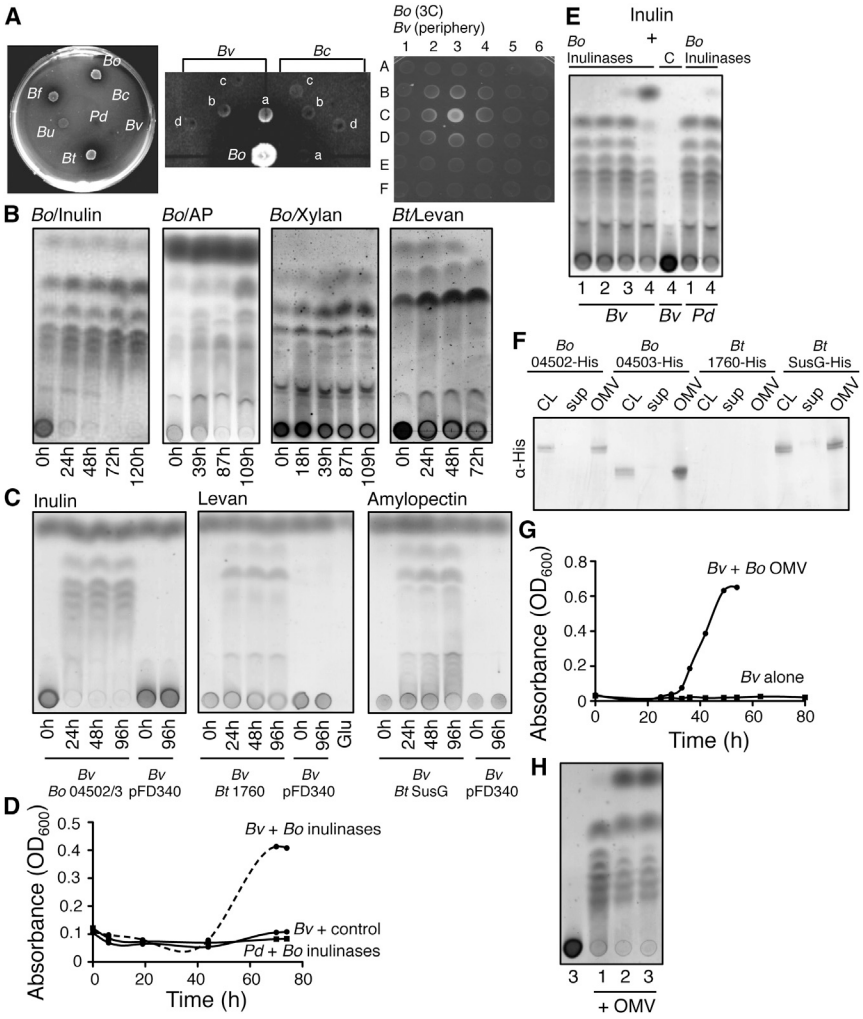

Figure 4. GH/PLs Serve as Public Goods through Secretion in Outer Membrane Vesicles

(A) Left: growth of utilizers on defined amylopectin agarose plates demonstrating amylopectin degraded zones surrounding *Bf*, *Bo*, and *Bt*, demonstrating extracellular release of GH. Middle: growth capabilities of recipient (*Bv*) and late recipient (*Bc*) plated at various distances (a, b, c, and d) from the producer *B. ovatus* on a defined amylopectin plate. *B. vulgatus* is only able to grow in the zone of amylopectin degradation, whereas late recipient, *B. caccae* does not (left). Right: growth of recipient *B. vulgatus* (spotted on all 36 spots except 3C) is dictated by its spatial proximity to the producer *B. ovatus* (spotted on 3C) on a defined inulin plate.

(B) TLC analyses of GH/PL activity in culture supernatants of PBP-liberating utilizer strains grown in indicated defined medium with extra polysaccharide added and incubated over time. The polysaccharide at the origin of the TLC is degraded with accumulation of PBPs.

(C) TLC analysis of extracellular GH/PL activity from recipient strains with GH/PL genes in *trans* or vector alone. Bacteria were cultured in defined glucose medium without polysaccharide as the GH/PL genes are expressed from a constitutive plasmid-borne promoter. Supernatants were harvested, filter sterilized, and diluted 1:1 with medium containing the indicated polysaccharide and incubated at 37°C over time prior to TLC analysis. The glucose at the top of the TLC is from the initial growth medium. Glu, glucose.

(D) Growth of *B. vulgatus* but not *P. distasonis* in defined inulin medium with purified BACOVA_04502 and BACOVA_04503 added to the medium. *B. vulgatus* does not grow with material purified from the vector only control.

(E) TLC analysis of the resulting media from the samples shown in (D), through the growth phases (1, lag; 2, lag; 3, late log; 4, stationary), demonstrating PBP consumption by *B. vulgatus*. "C" indicates *B. vulgatus* grown with material prepared from vector-only control. As this *B. vulgatus* with vector control material and *P. distasonis* did not grow in *Bo*-inulin CM, its time points correspond to the growth points of *B. vulgatus* with inulinases through the growth phases.

(F) Western immunoblot analysis of cell lysates (CL), supernatant (sup), or outer-membrane vesicles (OMVs) from wild-type transconjugants synthesizing His-tagged GH/PLs. BT_1760 was not tracked in this assay.

(G) Growth of *B. vulgatus* in defined inulin medium with added OMVs isolated from *B. ovatus* inulin CM. The OMVs were harvested from supernatant of *B. ovatus* grown to log phase so that the bacteria were actively growing at the time of harvest.

(H) TLC analysis of the resulting media from the samples shown in (G), through the growth phases (1, early; 2, mid; 3, late log), demonstrating PBP consumption by *B. vulgatus* during growth in OMV + inulin media. The first lane is *B. vulgatus* cultured inulin medium without OMV at the same time point as *B. vulgatus* with OMV at time point 3.

Data are representative of at least two independent experiments; one representative experiment is shown. See also Figure S3.

polysaccharide is due to the specific extracellular GH/PLs encoded by these genes in *trans* (Figure 4C).

If these GH/PLs are public goods, recipients should grow and utilize polysaccharide when the respective producer-derived GH/PL is added to the growth medium. To test this prediction, we purified recombinant producer derived PLs BACOVA_04502 and BACOVA_04503 (Figure S3B). Addition of these inulinases to inulin medium led to rapid degradation of the polysaccharide with accumulation of PBPs (Figure S3B) and supported the growth (Figure 4D) and utilization of PBPs (Figure 4E) by the recipient *B. vulgatus*, but not the nonrecipient *P. distasonis.*

Gram-negative bacteria communicate with and deliver cargo to other cells using various secretion mechanisms, one of which is the release of outer membrane vesicles (OMVs) [22, 23]. The producer-encoded GH/PLs (BACOVA_04502, BACOVA_04503, BT_1760, and BT_3698) each contain an N-terminal signal peptidase II (SpII) cleavage site, indicating that they are lipoproteins, most of which have been shown to localize to the outer surface of Bacteroidales species [21, 24]. We hypothesized that the secreted GH/PL public goods would be present in OMVs rather than the soluble fraction due to the lipid moiety of these molecules. To test this hypothesis, we His tagged these four proteins at their C termini and placed the recombinant plasmids in their background strains for protein localization studies. Western immunoblot analysis revealed that these GH/PLs are present in the OMV fraction of the supernatant (Figure 4F). OMVs isolated from *B. ovatus* grown to log phase in inulin medium were able to degrade inulin (Figure S3C) and supported the growth of recipient *B. vulgatus* in inulin (Figure 4G) with concomitant PBP depletion (Figure 4H). These data reveal that GH/PL public goods are carried

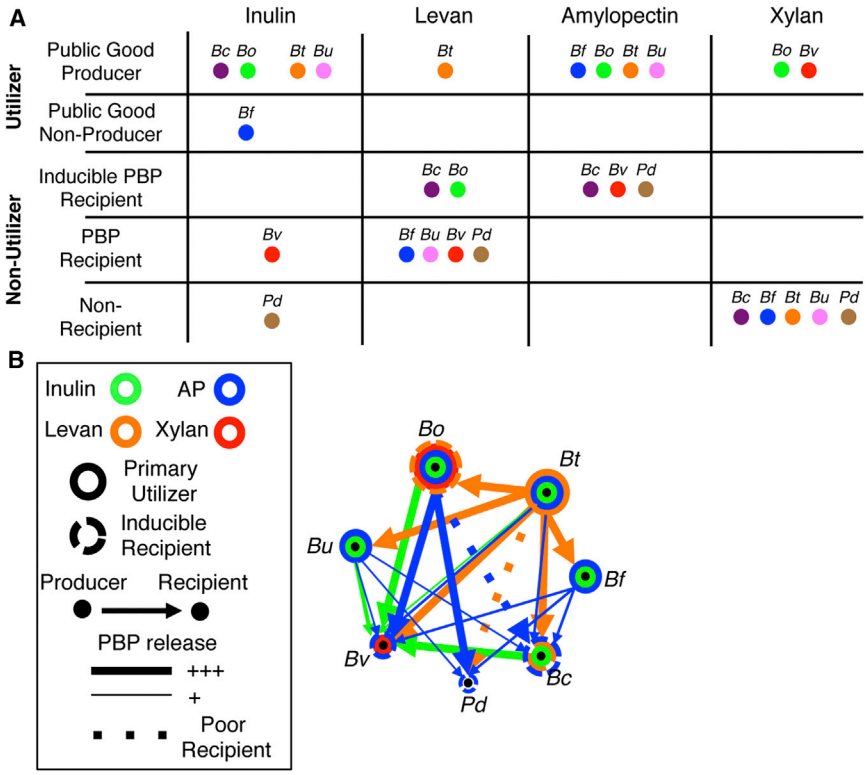

Figure 5. Ecological Classes and Network of Polysaccharide Utilization

(A) Schematic diagram designating Bacteroidales type strains to one of five ecological classes: utilizer/public good producer, utilizer/public good nonproducer, PBP public good recipient (inducible), PBP public good recipient (noninducible), and nonrecipient.

(B) A network of interactions based on PS utilization for Bacteroidales type strains.

See also Figure S4.

by OMVs and demonstrate a role for OMVs in ecological interactions among Bacteroidales.

Inducible Polysaccharide Utilization by Some Recipient Bacteria

Analysis of PBP consumption by recipients during growth in producer-derived CM revealed that, in some cases, residual polysaccharide was also degraded (Figure 2B). As we demonstrated that cell-free CM derived from producers contained GH/PL activity, we sought to determine the extent to which the producer-derived GH/PLs contributed to this degradation. We boiled CM to inactivate producer-derived GH/PLs and repeated the recipient growth experiments. Boiling of *B. ovatus* inulin CM abolished continued inulin degradation (Figure S4A), but did not affect PBP utilization and growth of recipient *B. vulgatus* (Figures S4B and S4C). Therefore, extracellular producer-derived inulinases accounted for the breakdown of inulin during *B. vulgatus* growth in this CM. In contrast, both *B. caccae* and *B. ovatus* grown in boiled *B. thetaiotaomicron* levan CM and *B. vulgatus* grown in boiled *B. ovatus* amylopectin CM showed continued robust depletion of polysaccharide not seen in control CM lacking bacteria (Figure S4C). Therefore, these recipients were induced by heat-stable producer-derived factors to catabolize the polysaccharide. This finding is consistent with the observation that under different experimental conditions, *B. vulgatus* has the ability to grow independently on amylopectin [25]. The ability to utilize polysaccharide was not the result of mutation as CM-induced recipients passaged through standard media were again unable to grow in polysaccharide defined media (Figure S4D). Together, these findings reveal a polysaccharide utilization network with five classes for each polysaccharide: utilizer/pubic good producer, utilizer/public good nonproducer, inducible polysaccharide-utilizing recipient, noninducible PBP recipient, and nonrecipient (Figures 5A and 5B).

Analysis of Fitness Benefits during Producer-Recipient Coculture

We next investigated the effects on fitness to both producers and nonutilizers (recipients and nonrecipients) during coculture. We used a producer auxotrophic for arginine, which allowed analyses of growth dynamics with and without an extrinsic growth limitation to the producer. In inulin, under producer-nonlimiting conditions, the fitness of *B. vulgatus* (inulin PBP recipient) increased during coculture with *B. ovatus*, whereas *P. distasonis* (a nonrecipient) did not (Figure 6A). In amylopectin medium, *B. vulgatus* and *P. distasonis*, which both utilize liberated PBPs and are induced to utilize polysaccharide, thrived in the presence of *B. ovatus*, whereas *B. caccae* (a poor recipient) again grew late (Figure 6B). Therefore, recipients, while unable to grow independently, can grow with an appropriate producer, but not outcompete the producer. We did not observe a decrease in fitness to the producer in any circumstance, suggesting that in these scenarios, the production of public goods may not itself be costly. Interestingly, however, during amylopectin coculture with recipients, an appreciable benefit to producer, as demonstrated by augmented growth compared to growth of producer alone, was observed (Figure 6B).

We next tested whether limiting the growth of the producer would allow a recipient to outcompete it. To investigate this possibility, we lowered the arginine concentration in the media so that the producer's growth was limited (Figure S5A) but sufficient to liberate PBPs (Figure S5B). Under these conditions, recipients dependent on PBPs (*B. vulgutus* in coculture with *B. ovatus* in inulin) and inducible PBP recipients (*B. vulgutus*, *P. distasonis*, and *B. caccae* in coculture with *B. ovatus* in amylopectin) all outcompeted the producer (Figure 6B) with robust PBP depletion and/or polysaccharide utilization by the recipients (Figure S5B). Together, these data demonstrate that in coculture, specific Bacteroidales members (recipient but not nonrecipient) can benefit from producer-derived public goods and that extrinsic limitation of the growth of the producer allowed recipients to dominate the population.

Bioinformatic Analysis of the Potential Production of GH/PL Public Goods among Gut Bacteroidales

Having established an ecological network of polysaccharide utilization in Bacteroidales type strains with these four

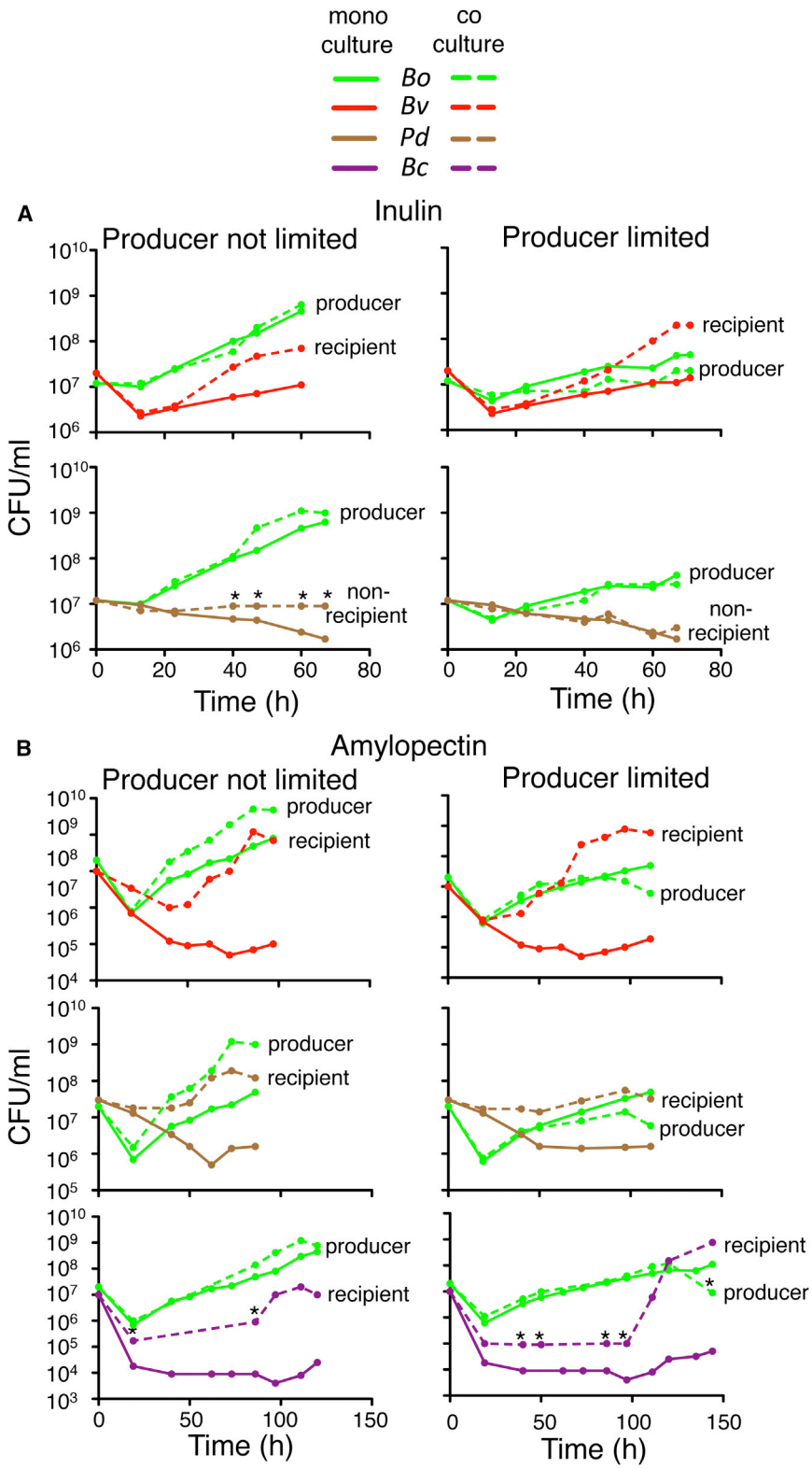

Figure 6. Fitness Assays of Polysaccharide-Utilizing and -Nonutilizing Strains in Coculture

Growth of type strains in coculture or monoculture in defined inulin medium (A) or defined amylopectin medium (B). The *B ovatus* strain is an arginine auxotrophic mutant, allowing for analyses under both growth limiting (low arginine, 1 μg/ml) and nonlimiting (high arginine, 16 μg/ml) conditions. Solid lines represent bacterial counts from monoculture experiments, whereas dotted lines represent bacterial counts from coculture experiments. Monoculture experiments for recipients were performed under low-arginine conditions only, as their growth is not affected by arginine; therefore, this growth curve is used for both producer-limited and not limited experiments. The limit of detection (indicated with * for strains that were below detection) for a given experiment is set at 2 logs below the total density of the culture. Data are representative of at least three independent experiments; one representative experiment is shown. Comparison of growth rates was as follows. Inulin producer nonlimited: *Bv* in monoculture (−0.08 ± 1.17) versus *Bv* in coculture (4.62 ± 0.05), p = 0.027. *Bo* in monoculture (5.58 ± 0.24) versus *Bo* in coculture with *Bv* (7.24 ± 2.4), p = 0.30, not significant (ns). Relative frequency producer: recipient (93.9% ± 2.4%:6.1% ± 2.4%). Inulin producer limited: *Bv* in monoculture (−0.08 ± 1.17) versus *Bv* in coculture (3.9 ± 1.2), p = 0.005. Relative frequency producer: recipient (5.3% ± 2.1%:94.7% ± 2.1%). *Bo* in monoculture (3.14 ± 0.37) versus *Bo* in coculture with *Bv* (3.08 ± 1.1), p = 0.48, ns. Amylopectin producer nonlimited: *Bv* in monoculture (−2.67 ± 0.73) versus *Bv* in coculture (4.69 ± 2.54), p = 0.02. *Bo* in monoculture (6.06 ± 1.13) versus *Bo* in coculture with *Bv* (9.34 ± 0.89), p = 0.02. Relative frequency producer: recipient (96% ± 1%:4% ± 1%). *Pd* in monoculture (−1.95 ± 1.07) versus *Pd* in coculture (1.42 ± 1.56), p = 0.05. *Bo* in monoculture (6.07 ± 1.13) versus *Bo* in coculture with *Pd* (9.88 ± 0.52), p = 0.045. Relative frequency producer: recipient (92% ± 2.7%:8% ± 2.7%). Amylopectin producer limited: *Bv* in monoculture (−2.7 ± 1.15) versus *Bv* in coculture (11.2 ± 4.1), p = 0.02. *Bo* in monoculture (6.4 ± 1.77) versus *Bo* in coculture with *Bv* (6.22 ± 1.08), p = 0.42, ns. Relative frequency producer: recipient (4.6% ± 2.7%:95.4% ± 2.7%). *Pd* in monoculture (−1.82 ± 1.23) versus *Pd* in coculture (5.84 ± 3.35), p = 0.87, ns. *Bo* in monoculture (6.37 ± 1.78) versus *Bo* in coculture with *Pd* (4.9 ± 0.23), p = 0.21. Relative frequency producer: recipient (15.7% ± 7.5%:84.4% ± 7.5%). Limits of detection precluded determination of growth rate of *Pd* in inulin coculture and *Bc* in amylopectin coculture. See also Figure S5.

polysaccharides, we next sought to examine the generalizability of these interactions. For the polysaccharides analyzed in this study, *B. caccae*, *B. vulgatus*, and *P. distasonis* were typically either recipients or nonrecipients, but rarely producers, whereas *B. thetaiotaomicron* and *B. ovatus* were more often producers (Figures 5A and 5B). Having established that SpII-containing GH/PLs can be secreted in OMVs as public goods, we used bioinformatics to determine whether there are differences in the number of SpII-containing GH/PLs among intestinal Bacteroidales. This analysis revealed that *B. caccae*, *B. vulgatus*, and *P. distasonis* type strains encode fewer predicted SpII-containing GH/PLs (28, 28, and 23, respectively) than do *B. thetaiotaomicron* and *B. ovatus*

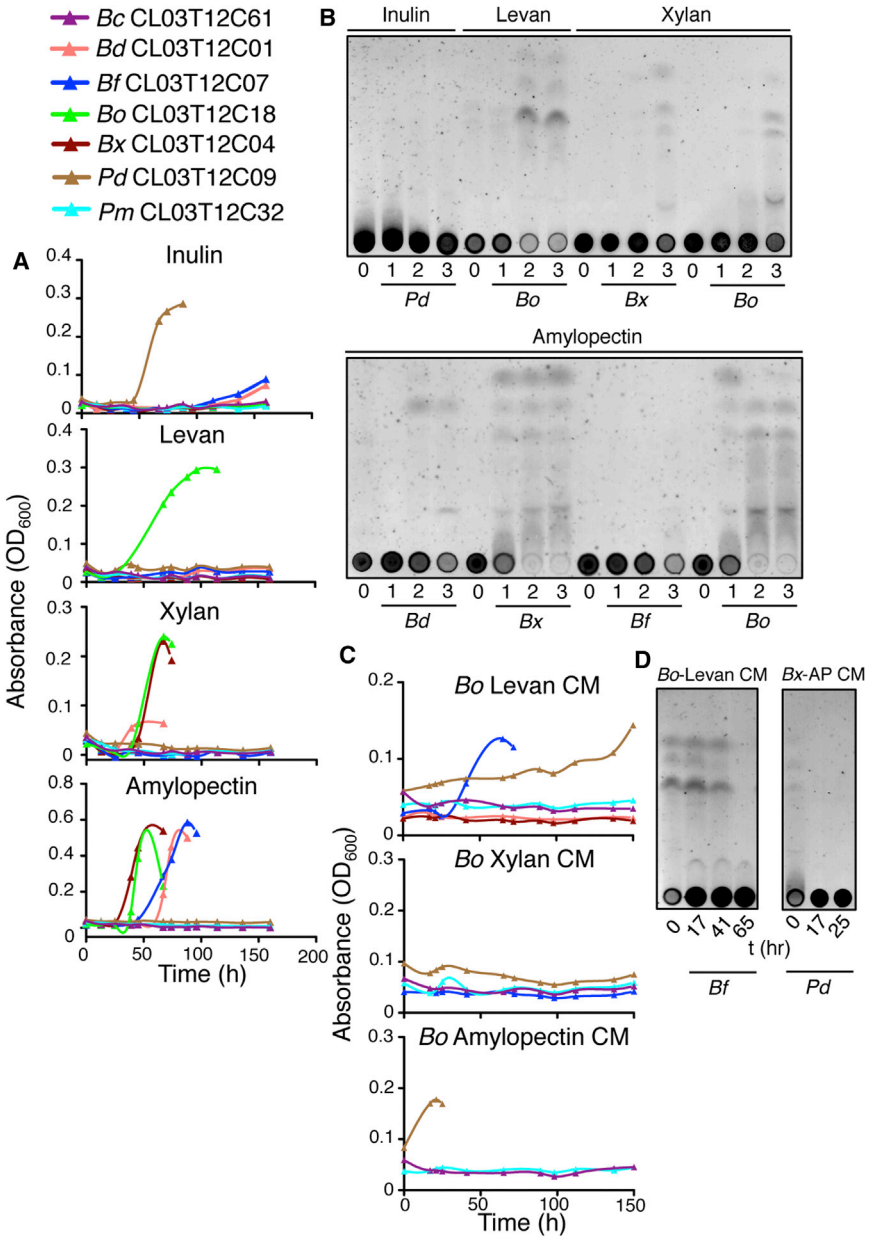

Figure 7. Polysaccharide-Based Ecological Relationships of Naturally Coresident Bacteroidales

(A) Growth curves of naturally coresident Bacteroidales strains from human subject CL03 in the four defined polysaccharide media.

(B) TLC analysis of PBP release during growth of CL03 strains in defined polysaccharide media.

(C and D) Growth (C) and TLC (D) analysis of PBP consumption by CL03-nonutilizing strains grown in the CM of primary utilizers, through the growth phases (1, early; 2, mid; 3, late log). Producer-derived CM was diluted with fresh polysaccharide containing defined media for recipient growth to assess inducible polysaccharide utilization. Therefore, lane 0 (undiluted producer CM) has less polysaccharide than the subsequent lanes.

Data are representative of at least two independent experiments; one representative experiment is shown.

type strains (54 and 80, respectively) (Tables S1 and S2). To determine whether this correlation could be expanded to the species level, we analyzed the genomes of 86 *Bacteroides* and *Parabacteroides* strains with draft or completed genomes. This analysis demonstrated a high level of uniformity in the number of SpII-containing GH/PLs among strains of a given species (Table S1), despite differences in the GH/PL gene repertoire within a species. These data suggest that roles as either producers or recipients are a conspecific trait with regard to polysaccharide utilization among the Bacteroidales. Certain species, such as *B. ovatus* and *B. cellulosilyticus*, are more likely to utilize polysaccharide with concomitant production of public goods, i.e., are PBP producers for a larger repertoire of polysaccharides, whereas *B. fragilis* and *P. distasonis* are more likely to be recipients in plant polysaccharide utilization webs in the gut ecosystem.

Polysaccharide Utilization Network among Highly Abundant Bacteroidales from a Natural Human-Derived Ecosystem

As the Bacteroidales type strains of this study were isolated from different individuals, we sought to determine whether these public goods-based interactions occur in coresident strains. We studied seven Bacteroidales species all cocolonizing a healthy human subject at a minimum concentration of 10^8 cfu/g feces [9]. Both utilizers and nonutilizers were identified for each of the four polysaccharides (Figure 7A). Among polysaccharide utilizers, individual members demonstrated PBP producer and nonproducer traits (Figure 7B). *B. ovatus* CL03T12C18 utilized levan, xylan, and amylopectin and liberated significant amounts of PBPs during growth in each of these polysaccharides. Conversely, while *P. distasonis* CL03T12C09 was able to grow in inulin and *B. fragilis* CL03T12C07 in amylopectin, these strains did not liberate any detectable PBPs. In addition, these data confirm that growth on a particular polysaccharide is not a species-wide property and support the bioinformatics analysis that certain species such as *B. ovatus* are producers that utilize many polysaccharides with concomitant PBP release compared to *B. fragilis* and *P. distasonis*.

To determine whether producer-recipient relationships occur among these naturally coresident strains, we monitored growth of nonutilizers in the CM of two PBP-liberating utilizers, *B. ovatus* CL03T12C18 grown in both levan and xylan and *B. xylanisolvens* CL03T12C04 grown in amylopectin. As observed in the type strains, xylan PBP did not support the growth of any nonutilizer (Figure 7C), whereas levan and amylopectin CM supported the growth of some nonutilizers (Figures 7C and 7D), identifying PBP recipients and nonrecipients. TLC analysis showed that *B. fragilis* CL03T12C07 grew in *B. ovatus* CL03T12C18 levan CM and *P. distasonis*

CL03T12C09 grew in *B. xylanisolvens* CL03T12C04 amylopectin CM until the PBPs were consumed (Figures 7C and 7D). These data reveal that abundant, coresident strains of Bacteroidales derived from a natural human gut ecosystem form a network for polysaccharide utilization based on the production of public goods.

Discussion

This study reveals a complex polysaccharide utilization network among the Bacteroidales and illuminates an important facet of the communal lives of the most abundant Gram-negative bacteria of the human intestine. Although metabolic webs are known to exist between members of the intestinal microbiota [26], previously described interactions are based on metabolic byproducts that are neither public nor common goods [26] and occur between phylogenetically distant members [26–28]. In contrast, public good production by Bacteroidales, which may have evolved for cooperation among clonemates, has resulted in a complex polysaccharide utilization network that has the potential for exploitation by recipients. In this network, trait variation exists among polysaccharide utilizers in that certain members liberate public goods, while others do not. This difference is likely due to the synthesis of distinct surface GH/PL among Bacteroidales species for utilization of the same polysaccharide [29]. Therefore, public good-producing and nonproducing strategies of polysaccharide utilization have simultaneously evolved among highly abundant, coresident Bacteroidales members. While this study focused on characterizing the complex network of interspecific interactions, it will be interesting to determine how the production of public goods balances the trade-offs of intra- versus interspecific competition and/or cooperation among the Bacteroidales.

At present, we do not know how the members of the human intestinal microbiota are spatially arranged. It is tempting to speculate that public goods-based polysaccharide utilization leads to the formation of spatially organized groups of producers and coevolved recipients, especially in the lumen, where host glycan foraging does not occur. Scaffolding provided by particulate matter has been suggested to be important for public goods-based dynamics in nonclonal, but closely related socially cohesive groups of bacteria in the ocean [12]. In the human gut, insoluble substrates have been demonstrated to support a specialized microbiota [30], and these insoluble substrates may serve as a scaffold to spatially organize these public goods-based interactions not only between the Bacteroidales, but also Firmicutes, and other less abundant members of this ecosystem.

A more comprehensive picture is emerging to explain how Bacteroidales species stably coexist at high densities over time. One strategy is the utilization of different polysaccharide substrates, therefore avoiding direct competition for carbon sources [15, 19, 31]. Another complementary strategy, not mutually exclusive to the first, revealed in this study, is the existence of polysaccharide public goods-based interaction networks where certain individuals can persist on carbon sources not supported by their own genes. This polysaccharide utilization network is consistent with the Black Queen hypothesis, which proposes that closely related bacterial species in communities form interdependent interactions marked by the loss of shared diffusible functions [32, 33], which in this network would be the secreted GH/PLs. In addition, reciprocal beneficial relationships or partner feedback mechanisms where factors derived from recipients increase the fitness of the producer [34] have likely evolved within these naturally communities. Indeed, we observe that for certain polysaccharides, coculture of producers and recipients leads to an increase in producer fitness not seen when producers are cultured alone, suggesting the evolution of mutualistic interactions.

By using a hypothesis-based approach, we applied social evolutionary thinking to understand the ecology of abundant members of the human microbiota and revealed a complex network of polysaccharide utilization and characterized the cellular and molecular mechanisms of these interactions. Both hypothesis-based and unbiased approaches to studying social and ecological interactions among the intestinal microbiota will be important in advancing this field. Bioinformatic and experimental approaches, coupled with evaluation of the fitness of different individuals within a group, will facilitate our understanding of the ecological dynamics of the intestinal microbiota. Such networks should be considered in both ecological modeling and therapeutically modulating the microbiota for human benefit.

Experimental Procedures

Bacterial Strains

Bacteroidales type strains used in this study are *B. caccae* ATCC 43185, *B. fragilis* NCTC 9343, *B. ovatus* ATCC 8483, *B. thetaiotaomicron* VPI 5482, *B. uniformis* ATCC 8492, *B. vulgatus* ATCC 8482, and *P. distasonis* ATCC 8503. Coresident Bacteroidales strains isolated from human intestinal ecosystems were previously described [9], and those used in this study include *B. caccae* CL03T12C61, *B. dorei* CL03T12C01, *B. fragilis* CL03T12C07, *B. ovatus* CL03T12C18, *B. xylanisolvens* CL03T12C04, *P. distasonis* CL03T12C09 and *P. merdae* CL03T12C32, all of which coexisted in a human at a density of at least 10^8cfu/g feces.

Bacterial Culture

For growth in defined or conditioned media, bacteria were inoculated from supplemented brain heart infusion (BHIS) plates into basal medium (BS), cultured overnight to stationary phase, then diluted 1:10 in fresh BS and grown to mid-log phase. At mid-log phase, bacteria were pelleted by centrifugation and washed with sterile PBS and then inoculated in either defined or conditioned media. Carbohydrates used to supplement defined media include glucose (G7528, Sigma), levan (L8647, Sigma), amylopectin (10120, Sigma), xylan (X4252, Sigma), and inulin (OraftiHP, Beneo-Orafti group). Conditioned media were prepared by filter sterilization of supernatants from bacteria grown in defined media to late-log phase. For initial experiments, conditioned medium was harvested at all phases of growth and compared for their ability to support recipient growth (Figure S2). For growth of bacteria in conditioned media, harvested conditioned media was diluted 1:1 with fresh defined medium without carbohydrate. In some instances, filter-sterilized conditioned media were boiled before being diluted with fresh carbohydrate-free defined medium. All cultures were grown at 37°C under anaerobic conditions. Bacterial growth was quantified by optical density (OD_{600}) using 200 μl of bacterial culture in 96 well flat-bottom microtiter plates using a Powerwave spectrophotometer (Biotek).

Coculture Experiments

For bacterial coculture experiments, bacteria were grown as indicated for monoculture prior to addition to the defined media. Quantification and differentiation of the two species was performed by plating dilutions on BHIS, followed by replica plating onto defined glucose minimal plates, which do not support the growth of the *B. ovatus* Δ03533 arginine auxotrophic mutant. Any ambiguous colonies were confirmed using a previously described PCR to differentiate these species [9]. The limit of detection for an outcompeted species was set at two logs below the total density of the culture.

See the Supplemental Experimental Procedures for bacterial media, molecular cloning and mutational methods, TLC procedures, and bioinformatics analyses.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.10.077.

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